ANALYSIS OF LOW MOLECULAR WEIGHT CARBOHYDRATES OF TOBACCO BY HIGH PERFORMANCE ION CHROMATOGRAPHY

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SUMMARY

The aim of the present work is to develop a High Performance Ion Chromatography method for the qualitative and quantitative determination of both reducing and non-reducing low molecular weight carbohydrates in tobacco leaf.
Detection and identification of these compounds was carried out using a DIONEX 4000 Ion Chromatograph equipped with a pulsed amperometric detector. Columns used were HPIC-AS6A (150x4mm, 5µm particle size bed) or HPIC-AS6 (250x4mm, 10µm particle size bed) with guard columns HPIC-AG6A and HPIC-AG6, respectively. Both columns used amino modified polystyrene-divinylbenzene phases.
The amperometric detector uses a multistep potential wave form of three different voltages, each applied for a few milliseconds, resulting in alternating anodic and cathodic polarizations. This allows the amperometric detection of the $-CH_2OH$ functions of carbohydrates and the cleaning and reactivation of the electrode surface.
Sodium hydroxide solutions were used to improve the anion separation of carbohydrates which behave as weak acids with a pKa in the range 12-14.
Both isocratic and gradient elutions were run using different effluent flows in the range 0.5-1.0 ml/min. The sensitivity of the detector was also increased by adding a more concentrated solution of sodium hydroxide at the end of the separator column. This resulted also in a reduction of baseline drift.
Twenty-two carbohydrates were separated by isocratic elution and twenty-four by gradient elution.
Some chromatograms of standard compounds are shown, together with samples obtained from cultivars of Bright, Kentucky, Maryland, Paraguay, Burley, and Oriental tobaccos. Some mono- and disaccharides were also identified from their retention times. Some quantitative determinations for glucose, fructose, inositol, and saccharose were carried out for the above cultivars. Results were in the range 2-17%, 1-13%, 0.5-2%, 0.5-6%, respectively.

INTRODUCTION

Carbohydrates make up a large proportion of the leaf of many tobacco varieties, both as free or in the combined state, and are of great interest because of their relationship to leaf quality

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and smoke flavour. Many papers have been published concerning the analysis of low-molecular weight carbohydrates in tobacco leaf and in cigarette filler(1).
Some authors determine the total reducing sugars with colorimetric methods, others describe gas-chromatographic procedures using the trimethylsilyl derivatives or estimate the individual compounds by paper and thin layer chromatography(2-4).
Battard J.C. and Chauvett C. used the high performance liquid chromatography for rapid characterization of tobacco sugars(5).
The purpose of this work is to find the best conditions for detecting and determining as large a number as possible of both reducing and non reducing low-mulecular weight carbohydrates present in standard mixtures and to apply such conditions to the analyses of tobacco leaf and cigarette filler by the aid of the High Performance Ion Chromatography with pulsed amperometric detection.

EXPERIMENTAL

REAGENTS

The twenty-five carbohydrates (Merck for biochemistry) reported in table 2 were used without further purification to prepare the standard solutions, using distilled and purified in an ELGASTAT system water.
These solutions were kept at 4°C and were found to be stable for at least 15 days.
Solutions of 0.2N and 1.0N NaOH (Merck or Fluka reagent grade) were used.
All eluent solutions were prepared by bubbling helium at first through the water to prevent the carbon dioxide contamination, and were kept under a blanket of helium during elution.

APPARATUS AND PROCEDURES

A DIONEX BIO-LC Chromatograph equipped with:
- a gradient pump module, operating up to 4000 p.s.i.;
- a Dionex Eluent Degas Module with Eluant Container set;
- a Pulsed Amperometric Detector (PAD-2) containing a working gold electrod;
- a sample injection valve with a 50µl sample loop;
- a Dionex DQP-1 post column pumping sistem;
- ON-GUARD RP filters;
- HPIC-AS6 column with amino modified polystirene-divinylbenzene phase (250x4mm, 10µm particle size bed) protected by a guard column HPIC-AG6 (70x4mm);
- HPIC-AS6A column (150x4mm) with amino modified polystirene-divinylbenzene phase, 5µm particle size bed, protected by a guard column HPIC-AG6A (50x4mm);
- a Varian 1200 recorder.

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OPERATING CONDITIONS
Temperature: room-temperature in the range 18-23°C;
PAD operating parameters: E_1=0.10V ; E_2=0.60V; E_3=-0.80V
$T_1=T_2=T_3$=300msec;
Meter scale: 10KnA;
Chart speed: 1.0cm/min;

For isocratic elutions:
Flow rate: 0.6ml/min;
Eluent: NaOH 0.1N or H_2O(65%)+NaOH 0.2N(35%);

For gradient elutions:
Flow rate: 1.0ml/min;
Gradient program:

TIME (min)	WATER (%)	NaOH(0.2N) (%)
0	100	0
5	100	0
8	85	15
16	85	15
46	10	90
50	10	90
51	100	0

Post column reagent: NaOH 1N at flow rate of 1ml/min.

RESULTS

Carbohydrates behave as weak acids with a pKa in the range 12-14. They are present in the anionic form in solutions with a pH>12 and can be retained and separated on anion exchange columns. Carbohydrates are also easily oxidized on the surface of noble metal electrode such as gold. For these reasons the anion exchange chromatography with pulsed amperometric detection represents a choice method providing many advantages, as specificity, selectivity and sensivity(6).
The pulsed amperometric detector uses a multistep potential wave form of three different voltages, each applied for a few hundreds of milliseconds, resulting in alternating anodic and cathodic polarizations. The first positive voltage allows, through an oxidating reaction, the amperometric detection of the $-CH_2OH$ functions of carbohydrates; the second higher positive potential cleans electrochemically the electrode surface, while the third negative voltage reduces gold oxide back to gold.
With such a detector only molecules having oxidable functional groups can be detected and therefore only compounds with amino or sulfide groups can interfere, while carboxylic acids and

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most of the inorganic species do not. The molecules containing carbonyl functions are not oxidized at the operating conditions followed in the present work(7,8).
Furthermore the anion exchange chromatography allows a good separation of a large number of carbohydrates, from monosaccharides to low molecular-weight polysaccharides, while non-ionic and basic compounds, even if oxidizable, are not retained and elute in the void volume without any interference.
It is still possible to get a high sensitivity: amounts in the range 1-50ng are easily detected (table 1). In the gradient elutions the sensitivity is increased adding a more concentrated solution of sodium hydroxide (1.0N) at the end of the separator column, keeping relatively constant, in this way, the pH in the detector cell and achieving also the lower drift of the baseline. It must still be pointed out that carbohydrate degradation in high pH solutions has not been observed since these reactions are slow on the time scale of chromatography.
The chromatographic parameters of 25 carbohydrates are given in table 2. The retention times and the values of k' were obtained under isocratic conditions. The retention times under gradient conditions are relative to rhamnose.
Twenty-two out of twenty-five carbohydrates were separated and identified by isocratic elution and twenty-four out of twenty-five by gradient elution. The chromatograms are shown respectively in Figures 1 and 2.

TABLE 1
DETECTION LIMITS FOR SOME SUGARS IN TOBACCO

SUGARS	DETECTION LIMITS (ng)	SUGARS	DETECTION LIMITS (ng)
Inositol	1.0	Xylitol	15.0
Glucose	4.0	Sucrose	50.0
Fructose	4.0	Arabinose	15.0
Galactose	5.0	Melibiose	25.0
Xylose	2.5		

ANALYSES OF TOBACCO

The tobacco leaves or the cigarette filler were ground and amounts of mg 100 of tobacco powder were weighed. The samples were shaken twice with 50ml of distilled and purified water and both extracts were combined and filtered. The solution was then injected through an ON-GUARD RP filter into the sample injection valve of the chromatograph using a 50µl sample loop, not later than eight hours after the extraction, because the solutions of carbohydrates extracted from tobacco were not stable even at low temperature.

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TABLE 2
CHROMATOGRAPHIC PARAMETERS

PEAK NUMBERS	SUGARS	T_r[a] (min)	k'[b]	t_R[c]
1	Inositol	1.707	0.104	0.188
2	Xylitol	1.888	0.221	0.205
3	Sorbitol	2.230	0.442	0.259
4	Mannitol	2.450	0.585	0.295
5	Trehalose	2.621	0.695	0.330
6	Fucose	2.621	0.695	0.580
7	2-Deoxy-D-Ribose	2.887	0.867	0.688
8	2-Deoxy-D-Galactose	3.040	0.966	0.768
9	Rhamnose	3.204	1.072	1.000
10	2-Deoxy-D-Glucose	3.559	1.302	1.125
11	Arabinose	3.559	1.302	1.152
12	Galactose	3.984	1.577	1.286
13	Lyxose	4.225	1.733	1.393
14	Glucose	4.225	1.733	1.438
15	Xylose	4.298	1.780	1.509
16	Fructose	4.625	1.992	1.554
17	Allose	4.987	2.226	1.643
18	Ribose	5.165	2.341	1.744
19	Melibiose	5.406	2.497	1.744
20	Sucrose	6.766	3.376	1.955
21	Lactose	7.206	3.661	2.321
22	Cellobiose	9.670	5.255	2.723
23	Gentiobiose	10.181	5.585	2.795
24	Raffinose	11.604	6.506	2.893
25	Maltose	15.992	9.344	3.554

[a]T_r=retention times in isocratic elution; [b]$k' = (t_- - t_-)/t_-$, where t_-=retention times of carbohydrates and t_-=retention time of eluent in isocratic elution; [c]t_R=retention times relative to rhamnose in gradient elution

RESULTS AND DISCUSSION

Several samples, obtained from different cultivars, were assayed. Typical chromatograms of some main cultivars are shown in figures 3-7. Some mono and disaccharides were identified from their retention times; the main were inositol, glucose, fructose, sucrose and galactose, the latter only in samples of Burley. Some quantitative determinations of glucose, fructose, inositol and sucrose were carried out for cultivars of Oriental tobaccos, Bright and Kentucky. The range of concentrations are given in table 3.

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In order to check the range of linearity of the detector response a linear calibration graph was obtained for each of the four carbohydrates by plotting peak areas vs. concentrations with correlation coefficients in the range 0.99939-0.99996.

TABLE 3
RANGE OF CONCENTRATIONS OF SOME SUGARS IN TOBACCO CULTIVARS

Cultivars	Inositol (%)	Glucose (%)	Fructose (%)	Sucrose (%)
Oriental	0.69÷1.70	2.0÷ 9.5	2.4÷ 8.5	traces
Bright	0.72÷1.60	3.2÷17.0	4.9÷12.2	0.5÷6.0
Kentucky	1.20÷1.40	0.5÷ 3.0	1.3÷ 2.5	traces

The linearity of the responses was confirmed using the rhamnose as internal standard. Linear calibration graphs were obtained by plotting the ratio A_c/A_r vs. concentrations, were A_c is the peak area of each carbohydrate and A_r is the peak area of rhamnose. The correlation coefficients were in the range 0.99984-0.99999. Through the described procedures anion exchange liquid chromatography and pulsed amperometric detection proved to be efficient instruments for the separation, the identification and the quantitative determination of low-molecular weight carbohydrates in tobacco leaves and in cigarette filler.
Other aspects of our study, such as the identification of some unknown peaks present in the report chromatograms and the quantitative determination of other carbohydrates, present in lower concentrations, are currently being studied.

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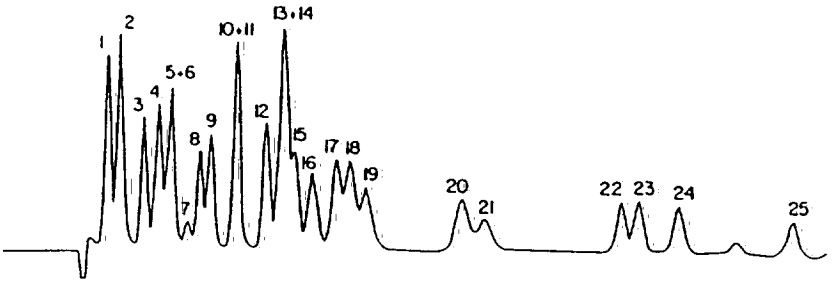

Fig. 1 - CHROMATOGRAM OF A STANDARD MIXTURE OF CARBOHYDRATES SEPARATED BY ISOCRATIC ELUTION. THE NUMBERS OF THE PEAKS CORRESPOND TO THE COMPOUNDS REPORTED IN TABLE I.

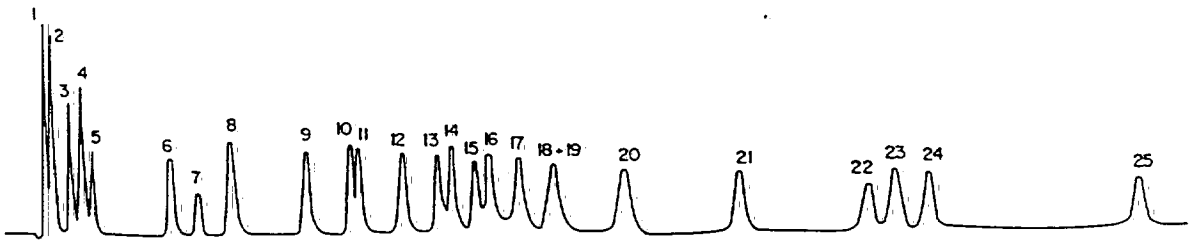

Fig. 2 - CHROMATOGRAM OF A STANDARD MIXTURE OF CARBOHYDRATES SEPARATED BY GRADIENT ELUTION. THE NUMBERS OF THE PEAKS CORRESPOND TO THE COMPOUNDS REPORTED IN TABLE I.

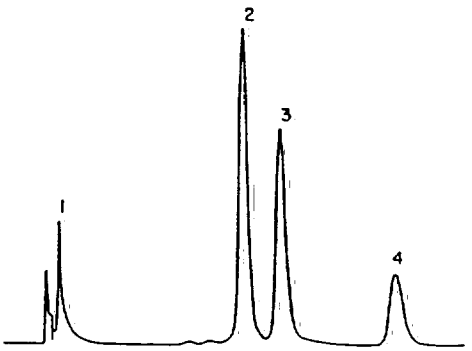

Fig. 3 - BRIGHT - ISOCRATIC ELUTION. METER SCALE: 10 μA

1: INOSITOL; 2: GLUCOSE;
3: FRUCTOSE; 4: SUCROSE

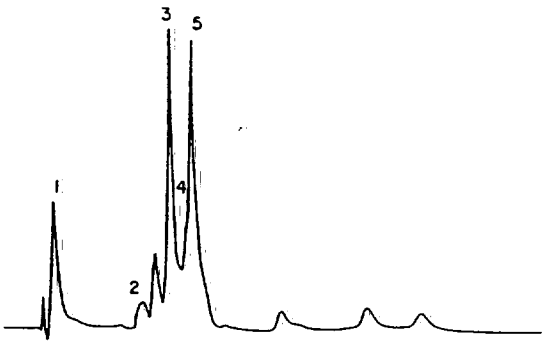

Fig. 4 - XANTY - ISOCRATIC ELUTION. METER SCALE: 10 μA

1: INOSITOL; 2: ARABINOSE; 3: GLUCOSE
4: XYLOSE; 5: FRUCTOSE

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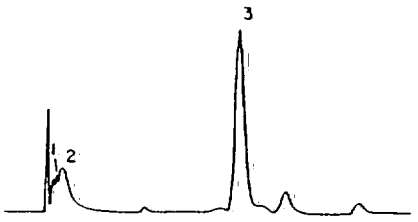

Fig. 5 - BURLEY - ISOCRATIC ELUTION. METER SCALE: 10 μA
1: INOSITOL; 2: XYLITOL; 3: GALACTOSE

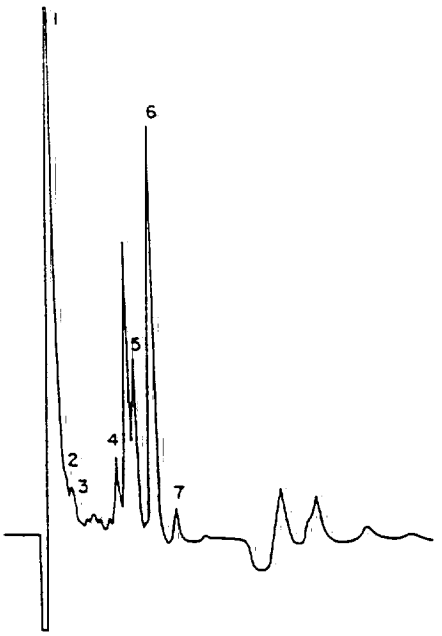

Fig. 6 - MARYLAND - ISOCRATIC ELUTION. METER SCALE: 3 μA
1: INOSITOL; 2: TREHALOSE; 3: 2-DEOXY-D-RIBOSE;
4: ARABINOSE; 5: GLUCOSE; 6: FRUCTOSE; 7: MELIBIOSE

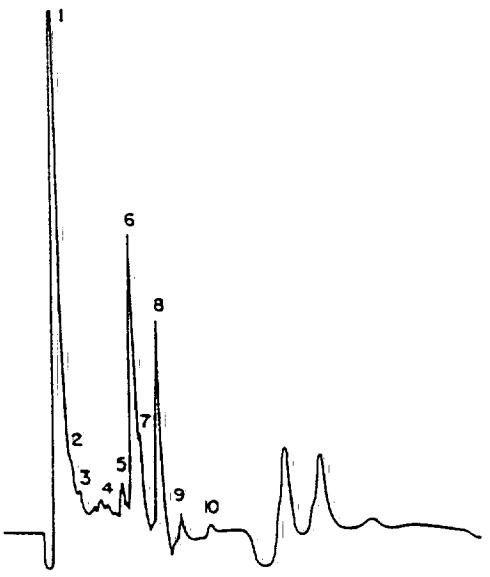

Fig. 7 - KENTUCKY - ISOCRATIC ELUTION. METER SCALE: 3 μA
1: INOSITOL; 2: SORBITOL; 3: MANNITOL; 4: RAMNOSE;
5: ARABINOSE; 6: GLUCOSE; 7: XYLOSE; 8: FRUCTOSE;
9: MELIBIOSE; 10: SUCROSE

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